Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 5166–5170, July 1988
Genetics

Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1

(genome rearrangement/herpesviruses/Cre–*lox*/metallothionein)

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Communicated by Robert A. Weinberg, March 28, 1988

ABSTRACT **The Cre protein encoded by the coliphage P1 is a 38-kDa protein that efficiently promotes both intra- and intermolecular synapsis and recombination of DNA both in *Escherichia coli* and *in vitro*. Recombination occurs at a specific site, called *lox*, and does not require any other protein factors. The Cre protein is shown here also to be able to cause synapsis of DNA and site-specific recombination in a mammalian cell line. A stable mouse cell line was established that expresses the Cre protein under the control of the Cd^{2+}-inducible metallothionein I gene promoter. DNA recombination was monitored with DNA substrates containing two directly repeated *lox* sites. One such substrate is a circular plasmid with two directly repeated *lox* sites (lox^2) flanking a marker gene and was introduced into cells by $Ca_3(PO_4)_2$ transformation. As a second substrate we used a pseudorabies virus (a herpesvirus) containing a lox^2 insertion designed to provide a sensitive detection system for recombination. In both cases, site-specific recombination *in vivo* is dependent on the presence of the Cre protein and occurs specifically at the 34-base-pair *lox* sites. These results demonstrate the controlled site-specific synapsis of DNA and recombination by a prokaryotic protein in mammalian cells and suggest that Cre-mediated site-specific recombination may be a useful tool for understanding and modulating genome rearrangements in eukaryotes.**

The processes governing DNA recombination in mitotic mammalian cells have been the subject of intense investigation in recent years. Recombinational events have been shown to be important in a variety of both normal and abnormal processes in mammalian cells. Mitotic recombination plays a central role in the development and function of the immune system. DNA recombination is also likely to be involved in both gene amplification events leading to drug resistance and the generation of the chromosomal translocations associated with some lymphomas and leukemias—for example, those in Burkitt lymphoma (1). The mechanism(s) by which recombination occurs in mammalian cells is poorly understood. Therefore, the introduction of a well-characterized site-specific recombination system into mammalian cells may provide some insight into certain aspects of recombination. The constraints imposed on DNA synapsis of chromatin structure, for example, could be one such concern. A site-specific recombination system would also be useful in directed manipulation of the eukaryotic genome.

The Cre–*lox* site-specific recombination system of coliphage P1 is particularly simple and well characterized (2–4). A single 38-kDa protein, Cre, is both necessary and sufficient to catalyze recombination between two *lox* sites, each of which is 34 bp in length. Recombination can occur between directly repeated sites on the same molecule to excise the intervening DNA segment or between inverted *lox* sites to give inversion of the intervening DNA segment. Both intra- and intermolecular recombination is catalyzed by Cre with either supercoiled or linear DNA. Recently we have shown that the Cre–*lox* system can function efficiently in yeast to cause recombination on chromosomes present in the nucleus (5). We now demonstrate that this site-specific recombination system also functions in a mammalian cell.

MATERIALS AND METHODS

Plasmids. The Cre expression plasmid pBS31 and its parent, pBMTx (obtained from G. Pavlakis, Frederick Cancer Research Facility), have been described (5, 6), as have pRH43 (7), pBS64, and pBS109 (8). M13mp11 neoΔS10, which contains an internal fragment of the kanamycin-resistance gene (*kan*^{r}) from transposon 5 (Tn*5*), was used for DNA hybridization analysis and was the gift of N. Sternberg (DuPont). Plasmids and the replicative form of M13 were prepared by standard techniques (9).

Mammalian Cells and Viruses. The swine kidney cell line PK15, the Becker strain of pseudorabies virus (PRV-Be), pseudorabies virus 42 (PRV42), and PRV42::pBS64 have been described (8), as has the procedure for the black-plaque assay (10). The mouse cell line C-127 (11) was transformed with the bovine papilloma virus-based vectors pBS31 and pBMTx [both containing the metallothionein I (MT-I) gene promoter] to establish cell lines 55 and 3C-5, respectively. The cell lines were obtained after colony purification of transformed cells from foci generated after DNA transformation (12). Southern blot analysis indicated that each cell line contained at least 60 extrachromosomal copies of the indicated plasmid, as anticipated (13).

DNA Transformation. $Ca_3(PO_4)_2$-mediated DNA transformation of mammalian cells was exactly as described by Lin *et al.* (14). Transient DNA transformation was performed in the absence of carrier DNA with 0.5 μg of pRH43 per 10-cm diameter dish containing 5×10^5 cells of the indicated cell line.

Analysis of DNA and RNA. Either total cellular DNA (15) or Hirt supernatant DNA (16) was prepared from cells and then analyzed by Southern blots (5, 17). DNA was electrophoresed in 0.8% agarose gels containing Tris borate buffer and no ethidium bromide (9). PRV DNA was prepared from nucleocapsids (18). Polyadenylylated RNA was prepared (19, 20) and then analyzed by Northern blots (9). Restriction enzymes and topoisomerase I were obtained from Bethesda Research Laboratories and used as recommended by the supplier. Cre protein was the gift of Ken Abremski (DuPont) and was used as described (8, 21).

Antibody and Immunoblot Analysis. Antibody to Cre and immunoblot analysis have been described (5). Additional antibody to the Cre protein was produced at Hazelton Biotechnologies (Denver, PA) by injection of a New Zealand

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Abbreviations: PRV, pseudorabies virus; *kan*^{r}, gene encoding kanamycin resistance; MT-I, metallothionein I.

White rabbit with 1 mg of purified Cre. Protein extracts of cells were prepared as described for yeast (5) with the omission of Zymolyase treatment. Protein extract (50 μg per lane) was electrophoresed on an 8% polyacrylamide gel containing sodium dodecyl sulfate and electrophoretically transferred to nitrocellulose for detection of the 38-kDa Cre protein by using Cre-specific antibody and ^{125}I-labeled protein A (5). The amount of Cre in cell extracts was determined by densitometry of the 38-kDa Cre protein and a comparison to a titration series of a known amount of purified Cre electrophoresed in parallel on the same gel.

RESULTS

The phage P1 *cre* gene was placed under the control of the Cd^{2+}-inducible *MT-I* promoter on a plasmid vector containing the bovine papilloma virus replicon and transforming region (5, 6). The resulting plasmid, pBS31 (Fig. 1*A*), was introduced into the C-127 mouse cell line, and transformants were identified by their ability to form foci. Cell line 55 is a subclone of one such focus that contains ≈60 extrachromosomal copies of pBS31.

RNA blot analysis revealed that *cre*-specific RNA was induced ≈50-fold in cell line 55 after treatment of cells with 8 μM $CdCl_2$ for 4 hr (data not shown). Immunoblot analysis of cell line 55 also showed $CdCl_2$ induction of the Cre protein, as described in more detail below. To determine whether the Cre recombinase expressed in cell line 55 was functional, we used a recombination assay with plasmid pRH43 (Fig. 1*B*). Plasmid pRH43 contains two directly repeated *loxP* sites flanking the *kan*^{r} gene from Tn*5* (7). Recombination by Cre at the *loxP* sites results in excision of the *kan*^{r} gene from pRH43 as a 1.9-kilobase (kb) circular DNA molecule containing a single *Bgl* II site. Calcium phosphate-mediated DNA transformation was used to introduce pRH43 into 55. Two different induction protocols were used to elicit Cre expression. In the first protocol (protocol I), cells were transformed with pRH43 and induced 19 hr later with 8 μM $CdCl_2$ for 4 hr. Total DNA was prepared and analyzed 21 hr after removal of $CdCl_2$. In the second protocol (protocol II), cells were transformed with pRH43 and 38 hr later were induced with 20 μM $CdCl_2$ for 4 hr. Total DNA was prepared for analysis 2 hr after removal of $CdCl_2$. Southern blot analysis of total DNA digested with *Bgl* II was then used to search for recombinant DNA species containing the *kan*^{r} gene (Fig. 2*A*). In addition to the 4.3-kb nonrecombinant fragment (NR in Fig. 2), a faint band was present in pRH43-transformed Cre^+ cells induced by protocol I (lane 6) having the same mobility

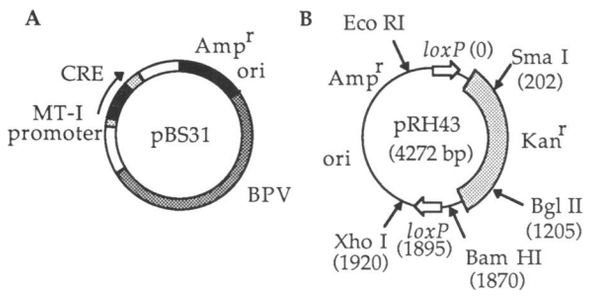

FIG. 1. Plasmids used in this study. (*A*) Plasmid pBS31 contains the *cre* gene under the control of the promoter from the mouse gene for MT-I. The arrow indicates the direction of transcription. Plasmid pBS31 also contains bovine papilloma virus (BPV) sequences that allow extrachromosomal replication of the plasmid in mammalian cells. (*B*) Map of pRH43. The positions of cleavage, in base pairs, for the indicated restriction enzymes are shown in parentheses, and the positions of the two directly repeated *lox* sites are represented by the open arrows (7). The shaded area represents the *kan*^{r} gene from transposon Tn*5*.

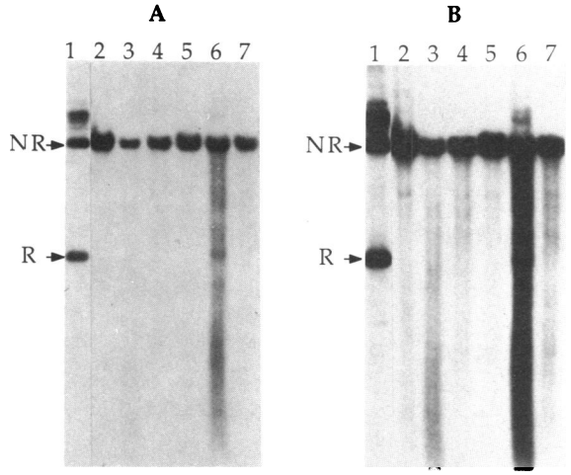

FIG. 2. Detection by Southern blot analysis of the excised *kan*^{r} fragment from pRH43. (*A*) Cells were transformed with pRH43 DNA by $Ca_3(PO_4)_2$ coprecipitation. Fresh medium was added, and 5 hr later cells were induced for 4 hr with 8 μM $CdCl_2$ (protocol I) or 24 hr later cells were induced for 4 hr with 20 μM $CdCl_2$ (protocol II). After induction, cells were washed with phosphate-buffered saline, and fresh medium was added (22). Total DNA (15) was prepared 30 hr after removal of the $Ca_3(PO_4)_2$ coprecipitate for all cultures, digested with *Bgl* II, and analyzed by Southern blotting with a *kan*^{r} probe. Lanes: 1, 1.3 ng of pRH43 DNA treated with Cre protein *in vitro* and digested with *Bgl* II; 2, uninduced C-127; 3, protocol I-treated C-127; 4, protocol II-treated C-127; 5, uninduced cell line 55; 6, protocol I-treated 55; 7, protocol II-treated 55. (*B*) A four-fold overexposure of the blot shown in *A*. NR, nonrecombinant (4.3-kb, linear fragment); R, recombinant (1.9-kb, linear fragment).

as the 1.9-kb fragment diagnostic of Cre-mediated recombination (R in Fig. 2). It was not present in uninduced cells (lane 5), nor was it present in protocol II-induced cells (lane 7), which were allowed only a short expression time. Parental Cre^- C-127 cells transformed with pRH43 did not exhibit this band under any condition (lanes 2–4), even after overexposure of the blot (Fig. 2*B*). A further 5-fold overexposure of the blot beyond that shown in Fig. 2*B* also did not reveal the recombinant 1.9-kb band in C-127 cells. The smear of hybridizing DNA from Cre^+ cells induced by protocol I is not understood but may indicate Cre-induced nicks with subsequent degradation by cellular enzymes. Curiously, this degradation was much more extensive 21 hr after Cd^{2+} removal than it was 2 hr after removal of Cd^{2+}, especially in the case of the Cre^+ cell line (compare lanes 3 and 4 with lanes 6 and 7).

To verify that the 1.9-kb band detected in Fig. 2 is indeed the predicted DNA species resulting from recombination between two *loxP* sites, we compared its restriction pattern to that of the authentic 1.9-kb recombinant molecule generated *in vitro*. Hirt supernatant DNA (16) was prepared from pRH43-transformed 55 cells subjected to induction protocol I, and the DNA was then, after digestion with various restriction enzymes, analyzed by Southern blotting with the *kan*^{r} gene as a probe. Enzymes that cleave on the *kan*^{r} side of pRH43 should reveal the 1.9-kb linear recombinant band, whereas digestion with *Eco*RI should leave an intact circular recombinant species. In all cases nonrecombinant pRH43 should be present as a 4.3-kb linear species (NR-lin). This is exactly the result obtained (Fig. 3*A*). Digestion with *Bam*HI, *Bgl* II, and *Sma* I (lanes 1, 2, and 4, respectively) all cleaved on the *kan*^{r} side of pRH43 and resulted in the predicted 1.9-kb band (R-lin). Digestion with *Eco*RI produced a band that comigrated with the open circular product of recombination (R-OC, lane 3).

We obtained additional evidence that recombination had occurred at the *loxP* sites of pRH43 by digesting the Hirt

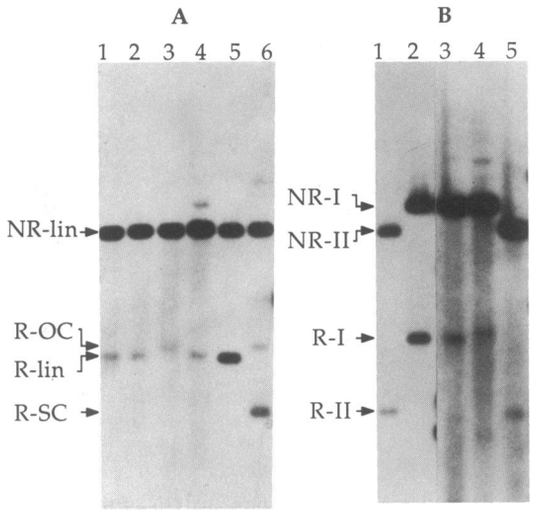

FIG. 3. Restriction mapping of the Cre-induced band. (*A*) Hirt supernatant DNA was prepared from cell line 55 cells transformed with pRH43 and induced according to protocol I. About 5% of the DNA obtained from a single plate was digested with *Bam*HI (lane 1), *Bgl* II (lane 2), *Eco*RI (lane 3), or *Sma* I (lane 4) and analyzed by a Southern blot using the *kan*^{r} probe. As markers, 0.13 ng of pRH43 was treated with Cre (lane 6) or treated with Cre and then digested with *Bam*HI (lane 5). (*B*) Hirt supernatant DNA prepared as described in *A* was digested with *Bgl* II (lane 3), *Xho* I (lane 4), *Bgl* II/*Bam*HI (lane 5). Markers were 0.13 ng of pRH43 treated with Cre and then digested with *Bgl* II (lane 2) or *Bgl* II/*Bam*HI (lane 1). NR, nonrecombinant; R, recombinant; lin, linear; OC, open circular DNA; SC, supercoiled DNA; NR-I and R-I, nonrecombinant (4.3 kb) and recombinant (1.9 kb) *kan*^{r} fragments linearized with *Bgl* II; NR-II and R-II, nonrecombinant (3.6 kb) and recombinant (1.2 kb) *kan*^{r} fragments linearized with *Bgl* II/*Bam*HI. The positions of open circular and supercoiled recombinant species were determined by treatment of the marker pRH43 with topoisomerase I prior to Cre-mediated recombination.

supernatant DNA with *Bam*HI and *Bgl* II (Fig. 3*B*). This resulted in the predicted 1.2-kb recombinant band (lane 5), whereas digestion with *Bgl* II alone gave a 1.9-kb band (lane 3). Digestion with *Xho* I did not cleave the circular *kan*^{r} species excised by Cre, as anticipated (lane 4). The predicted reciprocal product of recombination was also detected in these DNA preparations by using a probe specific for the *Amp*^{r} gene (data not shown). These results demonstrate that the Cre recombinase is expressed in 55 cells and is able to perform precise *lox*-specific recombination on pRH43 to excise a 1.9-kb circular molecule containing the *kan*^{r} gene.

A better understanding of Cre-mediated recombination in strain 55 was obtained by monitoring the generation of the excised fragment at various times after the 4-hr induction with 8 μM $CdCl_2$. In addition, we followed the induction of the 38-kDa Cre protein by immunoblot analysis using Cre-specific antibody (Fig. 4). At 1 hr after the induction period, there was only a small amount of Cre protein in these cells and there also was very little recombinant product. By 15 hr after induction, however, Cre had accumulated to near its maximal level, and 3.1% of the pRH43 introduced into 55 had been converted to recombinant product. The slight lag in recombination compared to accumulation of Cre protein observed at 5 hr may indicate either the inability of newly synthesized Cre to access pRH43 DNA or, alternatively, the need for a threshold level of Cre to be present for efficient recombination to occur. Certainly, the lag seen here contrasts with the rapid site-specific recombination (which takes only a few minutes) previously reported for the Cre–*lox* system *in vitro* (23). After 15 hr post-induction, no additional recombinant is formed.

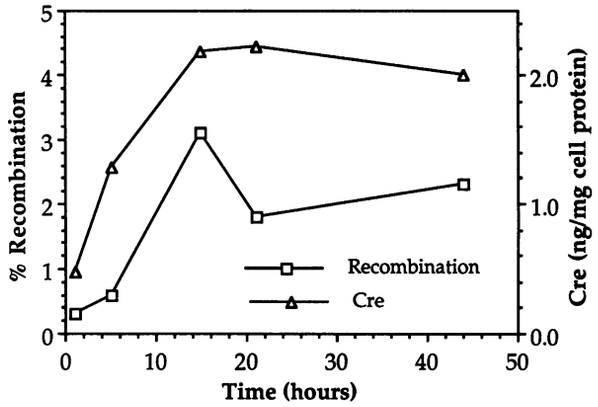

FIG. 4. Time course of Cre induction and Cre-mediated recombination. Cell line 55 cells were transformed with pRH43 DNA and induced with 8 μM $CdCl_2$ by protocol I. At the indicated times after removal of $CdCl_2$, total DNA was prepared from one plate of cells. A parallel plate was used to prepare a lysate for immunoblot analysis with Cre-specific antibody to determine the amount of Cre produced. The amount of recombination [% recombination = recombinant band/(recombinant band + nonrecombinant band) × 100] that had occurred was determined by densitometry of both the recombinant and nonrecombinant *kan*^{r} fragment present in a Southern blot of *Bgl* II-digested DNA, taking care that the relevant bands were within the linear response of the film.

Recently, we described the construction of a herpesvirus vector, PRV42::pBS64, that carries a 3.1-kb *lox*[2] insertion of bacterial plasmid DNA in the 150-kb genome of PRV, disrupting the *gIII* gene (ref. 8; Fig. 5). Precise recombination at the two *lox* sites results in reconstruction of the *gIII* gene (containing a single *lox* site), which produces a glycoprotein recognized by a specific antibody. Therefore, recombinant viruses make black plaques when treated with gIII-specific antibody and a horseradish peroxidase-linked second anti-

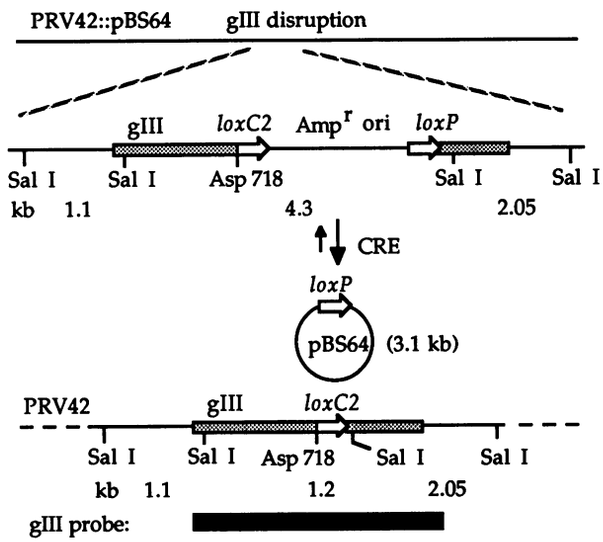

FIG. 5. Map of the *gIII* region of PRV42::pBS64. Insertion of the 3.1-kb plasmid pBS64 into the 150-kb genome of PRV42 by Cre-mediated recombination *in vitro* has been described (8). The distances between the *Sal* I sites are indicated below the map. The coding region of the *gIII* gene is indicated by the shaded box. The *loxC2* and *loxP* sites are indicated by the open arrows. Upon Cre-mediated recombination at the *lox* sites of PRV42::pBS64, black-plaque-forming PRV42 virus is produced which contains a reconstructed *gIII* gene. Conversely, recombination between PRV42 and pBS64 *in vitro* results in an integrative event to regenerate PRV42::pBS64. The *Nco* I fragment of pBS109 was used as a *gIII* probe in Fig. 6 and is indicated by the black box.

body, whereas nonrecombinant viruses form white plaques. Thus, the PRV42::pBS64 virus should provide a convenient assay for precise Cre-mediated recombination *in vivo*.

Strain 55 was infected with PRV42::pBS64, and the plaques obtained were subjected to the black-plaque assay (Table 1). Approximately one-fourth of the resulting plaques on cell line 55 exhibited a black, sectored phenotype indicative of recombination at the *lox* sites. There were no black or sectored plaques obtained after plating PRV42::pBS64 on the parental C-127 cell line, on 3C-5 (a C-127 transformant containing the bovine papilloma virus MT-I vector without the *cre* gene), or on the porcine PK15 cell line normally used to propagate PRV. Black-staining areas comprised roughly 10 to 30% of a plaque identified as sectored, suggesting that 10–30% of the virus in such a sectored plaque had reconstructed the *gIII* gene. Therefore, plate stocks of PRV42::pBS64 were prepared both on 55 and on C-127 with or without induction with 2 μM $CdCl_2$, and the resulting virus was plaqued on PK15 cells (Table 2). In experiment 1, virus passaged through the 55 cell line contained 3.3% recombinant virus. No recombinant virus was observed in the viral stock grown on the Cre^- C-127 cell line, in agreement with our previous observation that homologous recombination between *lox* sites in PRV42::pBS64 occurs at a frequency less than 1×10^{-5} (8). In experiment 2, almost 25% of the virus from PRV42::pBS64 plate stocks prepared on 55 cells showed a black-plaque phenotype indicative of recombination when assayed on PK15 cells. Incubation of the cells with 2 μM $CdCl_2$ did not stimulate the production of recombinant virus. Treatment of cells with higher concentrations of $CdCl_2$ resulted in cells refractory to productive PRV infection.

The differences in the amount of recombination seen between the two experiments may be due either to the state of the cells prior to PRV infection or to the multiplicity of infection. However, the amount of recombination detected was not induced by 2 μM $CdCl_2$ and was in fact somewhat reduced. Treatment with a low level of $CdCl_2$ may be insufficient to induce Cre production. The recombination events that were detected with PRV42::pBS64 could be due to a modest amount of Cre protein in the cell prior to infection. Alternatively, PRV may induce the MT-I gene in 55 cells and thus also the *cre* gene. Another herpesvirus, human cytomegalovirus, has in fact been shown to induce expression of the MT-I gene (R. Rüger, personal communication).

Precise recombination at the *lox* sites of PRV42::pBS64 predicts that the recombinants have a genomic structure identical to that of PRV42. PRV42 contains only a single *lox* site inserted into the *gIII* gene of PRV-Be and lacks the 3.1-kb insertion of pBS64 DNA present in PRV42::pBS64 (Fig. 5). Southern analysis of four black-plaque-forming recombinants obtained from the experiment described above showed that this is so (Fig. 6). Like PRV42, all four isolates (A–D) exhibited the 1.2-kb *Sal* I fragment diagnostic of Cre-mediated recombination (lanes 1–6). The equivalent *Sal* I fragment of PRV-Be was slightly smaller because it lacks the 41-bp insertion of DNA containing the *loxC2* site (8). We also determined that the *Asp* 718 site was retained in the four recombinants (data not shown). That a functional *lox* site is present in the recombinants was shown by their ability to undergo Cre-mediated recombination *in vitro* and the *lox-*

Table 1. Detection of sectored PRV::pBS64 plaques on a Cre^+ cell line

Cell line	Plaque morphology	
	Black/sectored	White
C-127 (Cre^-)	0	765
55 (Cre^+)	135	361
3C-5 (Cre^-)	0	351
PK15 (Cre^-)	0	2450

PRV42::pBS64 was plated on the indicated cell line, and the resulting plaques were analyzed by the black-plaque assay with the M1 monoclonal antibody to gIII (24) and a horseradish peroxidase-linked second antibody. PK15 is a swine kidney cell line that is the usual host for PRV (8). 3C-5 is a transformant of C-127 containing pBMTx, which is identical to pBS31 but lacks the *cre* gene (5, 6).

Table 2. Production of recombinant virus upon growth in a Cre^+ cell line

Cell line	$CdCl_2$, μm,	Plaque type of progeny virus	
		Black	White
	Experiment 1		
C-127 (Cre^-)	0	0	2106
C-127 (Cre^-)	2	0	1500
55 (Cre^+)	0	22	641
55 (Cre^+)	2	11	530
	Experiment 2		
55 (Cre^+)	0	56	138
55 (Cre^+)	2	76	325

The indicated cell lines were infected with PRV42::pBS64 at a multiplicity of 0.1 (experiment 1) or at a multiplicity of 0.02 (experiment 2). Induction with the indicated amount of $CdCl_2$ was for 5 hr prior to infection in experiment 1 and 11.5 hr prior to infection in experiment 2. Progeny virus were harvested when at least 80% of the cells appeared to be infected. Resulting virus stocks, after they were plated on PK15, were analyzed by the black-plaque assay, as described in Table 1.

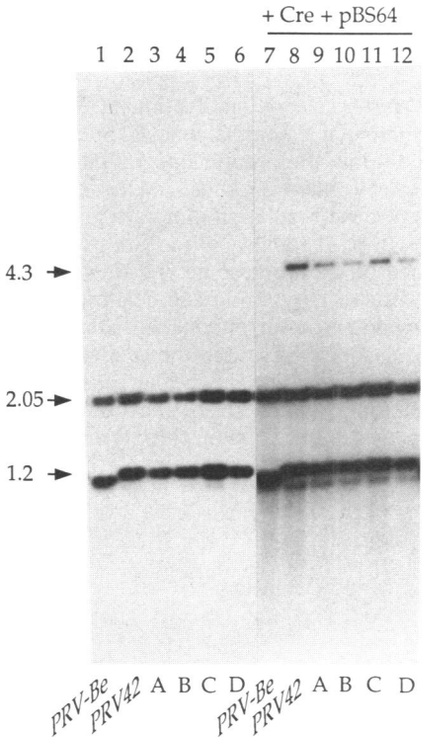

FIG. 6. Southern blot analysis of PRV recombinants. Four black-plaque-forming viruses (labeled A–D) from experiment 2 of Table 2 were plaque-purified, and nucleocapsid DNA (18) was prepared for Southern blot analysis and comparison to that of PRV-Be and PRV42. DNA was digested with *Sal* I only (lanes 1–6) or incubated with Cre and pBS64 *in vitro* prior to digestion with *Sal* I (lanes 7–12). Integration of pBS64 into PRV42 results in the production of about 10% PRV42::pBS64 (8), which exhibits the diagnostic 4.3-kb *Sal* I fragment shown in Fig. 5. The presence of a functional *lox* site on PRV DNA is thus indicated by generation of this band after Cre-mediated recombination. The *gIII*-specific probe (the *Nco* I fragment of pBS109) and the sizes of the hybridizing bands corresponding to the *gIII* region are shown in Fig. 5.

containing plasmid pBS64 (Fig. 6, lanes 7–12). Intermolecular recombination of either PRV42 or one of the four recombinants with pBS64 yielded a characteristic 4.3-kb *Sal* I fragment. PRV-Be, which does not contain a *lox* site, generated no such recombinant fragment. We conclude that the recombinants obtained from virus passaged through the Cre^+ strain 55 have undergone precise Cre-mediated recombination in mammalian cells at the 34-base-pair *lox* sites.

DISCUSSION

We have constructed a mouse cell line that contains the Cre recombinase of phage P1 under the control of the inducible promoter of the mouse gene for MT-I. DNA introduced into these cells either by transformation or by viral infection is recognized as a substrate for Cre-mediated site-specific recombination at *lox* sites.

We anticipate that cell lines able to express the Cre protein, such as the one we describe here, will be useful in the construction of recombinant viruses with large genomes. Recently, we described an efficient method for introducing heterologous DNA into a herpesvirus vector and then retrieving it by using Cre-mediated recombination *in vitro* (8). That method is likely to be useful both in the mutational analysis of a particular gene and in the construction and characterization of herpesvirus-based vaccines. The *in vitro* method is not, however, easily adaptable to other viruses whose DNA is noninfectious, notably vaccinia (25). Our demonstration that Cre can work in a mammalian cell indicates that Cre-mediated shuttling of DNA cartridges is applicable to all viruses having a DNA form, including vaccinia.

Can Cre also cause recombination at *lox* sites located within the genome of a mammalian cell? Such recombination events would allow the precise integration and excision of DNA at a predetermined chromosomal locus, thus facilitating the analysis of gene regulation in a particular chromosomal context. However, the ability of the Cre protein to access a *lox* site placed on a chromosome and then to perform site-specific synapsis of DNA and reciprocal recombination may be highly dependent on surrounding chromatin structure and on the particular location within the genome of the *lox* site. Some regions of the genome may be inaccessible to a bacterial recombinase, for example. If so, the Cre–*lox* system may provide important information on chromosomal structure and its response to regulatory mechanisms in the cell.

We gratefully acknowledge the technical assistance of Carol Burns, the gift of M1 antibody from Harmut Hampl and Tamar Ben-Porat, the critical reading of the manuscript by Nat Sternberg and Gerry Jonak, and the careful typing of the manuscript by Penni Lockhart.

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